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UTILIZATION OF PHOSPHATES IN THE POSTGERMINATIVE DEVELOPMENT OF SPORES OF *BACILLUS MEGATERIUM*

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Halvorson and Church (1957) have suggested that one approach to an understanding of germination might be a comparison of the enzyme patterns of vegetative cells, dormant spores, and spores in different stages of germination. Similarly, we have long felt that a differentiation of germination from postgerminative development on the basis of nutritional requirements would give some insight into the changes that occur when the dormant spore becomes a rapidly metabolizing cell. We have defined germination as the spore's initial loss of heat resistance accompanied by increased metabolic activity; stainability with methylene blue; and loss of refractility. Postgerminative development (Levinson and Hyatt, 1956) was considered to include later stages in the transition of the germinated spore into a vegetative cell. Each of the morphological phases of postgerminative development (swelling, emergence, elongation, and cell division) is accompanied by a change in respiratory rate (Mandels *et al.*, 1956). When glucose is present, Mn^{++}, L-alanine, or heat stimulates spore germination, but added sulfur is necessary for postgerminative development (Hyatt and Levinson, 1957). Co^{++} and Ni^{++} do not affect germination, but these ions differentially inhibit stages in subsequent development.

The present report describes further differences in the requirements for germination and for postgerminative development. We have found that the pH range for optimal germination is lower than that for postgerminative development. Germination occurs under both aerobic and anaerobic conditions (confirming the findings of Roth and Lively, 1956), but further development does not take place in the absence of oxygen. We have substantiated the postulate (Hyatt and Levinson, 1957) that, although spores will germinate without the addition of phosphate, added phosphate is required for the transition from germinated spore to vegetative cell. Numerous inorganic and organic phosphate compounds support this postgerminative development, and some of these compounds are more readily utilized in the presence of Mn^{++}. A pyrophosphatase has been demonstrated in intact developing cells, and metal activation and pH data strongly suggest that more than a single enzyme system is operative. The presence of phosphatases acting on substrates other than pyrophosphate has also been considered.

MATERIALS AND METHODS

Spores of *Bacillus megaterium* strain QM B1551 were from the same pool of lyophilized spores used previously (Hyatt and Levinson, 1957). Briefly, they were harvested from phosphate-buffered liver broth (Foster and Heiligman, 1949), washed by centrifugation at 4 C, and dried from the frozen state. These spores had been maintained in the lyophilized state for about 18 months without detectable change in their ability to germinate and to undergo postgerminative development. On a dry weight basis (16 hr in vacuum oven at 50 C), these spores contain approximately 2.5 per cent total phosphorus (method of LePage, 1957). Heat shocked spores were used throughout (5 min immersion of a buffered suspension in a water bath at 50 C). When germinated spores were used, they were prepared by incubating a buffered spore suspension, with glucose (25 mM) and a sulfate source (0.4 mM), on a shaker for 120 min at 30 C.

Spore germination was estimated by a modification (Levinson and Sevag, 1953) of the method of Powell (1950). Germinated spores were differentiated from ungerminated spores by their stainability with methylene blue. Microscopic observation of stained preparations was also used in the separation of the morphological stages subsequent to germination.

Unless otherwise indicated, all reaction systems contained spores, 1 mg (4.2×10^8 spores) per ml; glucose, 25 mM; and ammonium acetate, 50 mM, as a nitrogen source. Sulfate (0.4 mM) was supplied as K_2SO_4, except that $MnSO_4$ was used when the effect of Mn^{++} was being

investigated. Sodium cacodylate (60 mM) was the principal buffer used in these experiments, but Tris [tris(hydroxmethyl)aminomethane], sodium barbital, KH_2PO_4, and glycylglycine (50 mM) were also used, where indicated. Phosphate sources were added at a concentration of 1.0 mM of phosphorus (e. g., sodium pyrophosphate was used at a concentration of 0.5 mM). Except for KH_2PO_4, inorganic phosphates were generally used as the sodium salts. All concentrations are final, after tipping or starting the reaction.

Oxygen consumption was measured at 30 C by conventional Warburg techniques, using 0.3 ml of glucose and sulfate in the sidearm; 1.5 mg of spores in 1.0 ml of buffer, and 0.2 ml of phosphate in the main chamber; and 0.2 ml of 10 per cent KOH in the center well. When phosphate was added at different time intervals, glucose, sulfate, and spores in buffer were placed in the main chamber and phosphate was tipped in from the sidearm.

Percentage utilization of various phosphates by developing spores was determined by estimation of the amount of phosphate remaining after incubation of the spores with pyrophosphate, glucose-1-phosphate, trimetaphosphate, tripolyphosphate, or orthophosphate. At various time intervals, aliquots of the remaining phosphate substrate were hydrolyzed in 1 N HCl at 100 C for 7 min, and the orthophosphate phosphorus resulting from this hydrolysis was measured by the method of Fiske and SubbaRow (1925).

Phosphatase activity was determined by estimation of production of orthophosphate from various phosphate compounds. Reaction systems contained germinated spores in buffer; glucose; sulfate; phosphate source; and, when used, inhibitor or metal activator. Incubation was carried out in 50-ml flasks on a shaker at 30 C. Controls containing spores, but not phosphate; or phosphate but not spores, were included in all experiments. At various time intervals, 1.5-ml aliquots were removed, and the reaction stopped by the addition of 0.15 ml of 100 per cent (w/v) trichloracetic acid. The precipitated protein was removed by centrifugation at 1 to 4 C and the supernatant tested for orthophosphate. Total hydrolysis of the added phosphate sources yields 31 μg of orthophosphate per ml.

RESULTS

Optimal pH levels for germination and for postgerminative development. At pH 7.0, in a phosphate-buffered medium, spores germinate and undergo postgerminative development, but at pH 6.0, although germination occurs, there is no subsequent development (Levinson and Hyatt, 1956). For the purposes of the present study, it was necessary to determine the pH range for postgerminative development, so that a distinction could be made between phosphate requirements on the one hand, and pH requirements on the other.

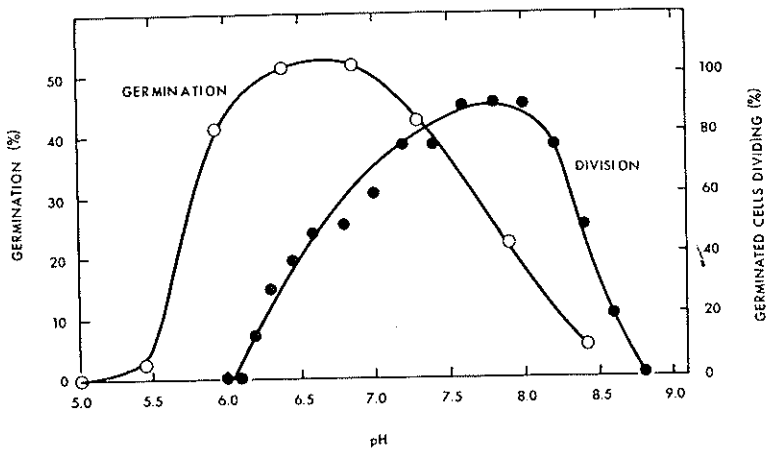

Figure 1. Effect of pH on germination and on postgerminative development of spores of *Bacillus megaterium*. All reaction systems contained glucose and phosphate buffer, 50 mM. Germination data taken at 180 min. For postgerminative development, spores were germinated at pH 6.8, aliquots adjusted to indicated pH levels, and K_2SO_4 added. Postgerminative development plotted as per cent of germinated spores which had divided at 440 min.

In phosphate buffer, containing glucose, spores were found to germinate in a broad pH range, with an optimum from 5.9 to 7.3 (figure 1). Since so few spores germinate at the higher pH levels, the spores used to determine the pH range for postgerminative development were preincubated for 2 hr at pH 6.8 to give 50 per cent germination. Aliquots of these preincubated spores were then adjusted to the desired pH with dilute HCl or NaOH. The pH was readjusted at hourly intervals. Without such readjustment, the pH dropped as much as 0.8 pH units during the 6 hr of incubation. Normal postgerminative development does not occur unless the pH is maintained over 6.4, and optimal development is between 7.5 and 8.0 (figure 1), where 90 per cent of the germinated cells divide. Below pH 6.4, the few cells which divide appear abnormal. An initial pH of 7.0 to 7.2 was used in all subsequent experiments, compromising between maximal germination and maximal postgerminative development.

Phosphate requirement for postgerminative development. Spores germinate in glucose in an acetate-buffered system (Levinson and Hyatt, 1956) with no added phosphate being required. Demonstration of a requirement for added phosphate in postgerminative development however, necessitated showing that, at a favorable pH (6.4 or higher) such development does not occur in the absence of phosphate. Buffers such as acetate, piperazine and Tris, initially at neutrality, do not maintain the proper pH level for postgerminative development. Tris, however, at a sufficiently high initial pH (7.6), maintains the level over 6.5, and although the percentage of germination is reduced, postgerminative development occurs, but only if phosphate is added. Barbital and glycylglycine actually inhibit the development of spores in the presence of phosphate. However, sodium cacodylate (dimethylarsonate) meets the requirements for a satisfactory buffer, as it is not inhibitory and spore suspensions are maintained within 0.3 pH units of

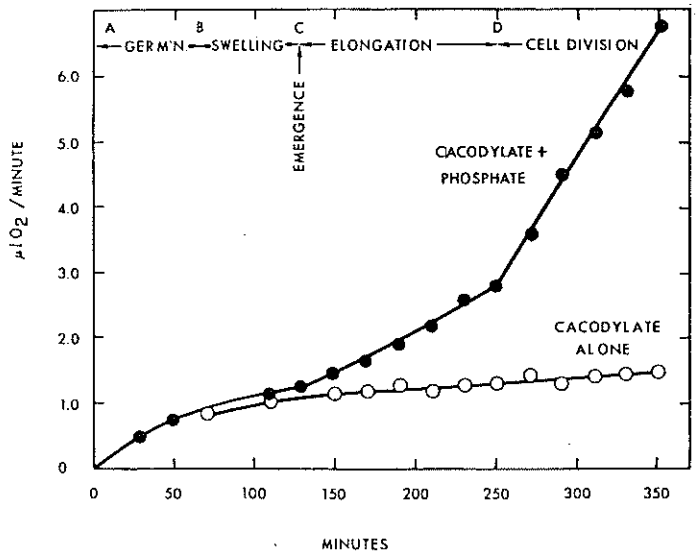

Figure 2. Effect of the addition of orthophosphate on the rate of oxygen consumption of spores of *Bacillus megaterium*. All reaction systems contained cacodylate buffer (pH 7.0), glucose, and K_2SO_4. Orthophosphate added at 1.0 mM. Developmental phases are indicated at the top of the figure as *A*, *B*, *C*, and *D*.

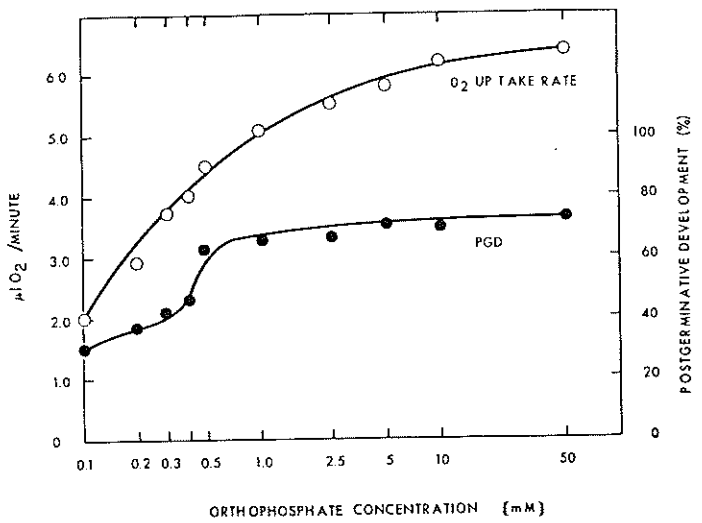

Figure 3. Effect of orthophosphate concentration on the rate of oxygen consumption, and on the postgerminative development (PGD) of spores of *Bacillus megaterium*. All reaction systems contained cacodylate buffer (pH 7.2), glucose, and K_2SO_4. Reaction time was 360 min.

an initial pH of 6.8 to 7.2, during 6 hr of incubation.

In cacodylate buffer, with no added phosphate, spores germinate, and some spores swell and crack, but over an extended period there is no further development. The low respiratory rate (figure 2, *B*) does not increase appreciably after 70 min when maximal germination has been attained. Addition of orthophosphate, 1.0 mM, to the cacodylate buffer, supports typical postgerminative development. The germinated spore swells, and at 130 min the spore coat cracks and emergence occurs (figure 2, *C*). The cells elongate, and at 250 min (figure 2, *D*) cell division begins. Changes in respiratory rate correspond to each of these developmental phases.[1]

A few cells emerge, elongate, and begin to divide at concentrations of orthophosphate between 0.1 and 0.4 mM. However, from 0.5 to 1.0 mM phosphate is necessary for a maximal number of cells to undergo at least one cell division (figure 3). Respiration rates are higher with further increase in phosphate concentration, although the percentage of postgerminative development is unaffected.

It is not necessary for phosphate to be present from the beginning of incubation, before the spores have germinated, in order for postgerminative development to occur. When phosphate is added at 50, 90, or 120 min to spores incubating in cacodylate buffer, glucose, and K_2SO_4, emergence occurs at 130 min, just as it does when phosphate is present from the beginning. When phosphate is added at 170 or 210 min, elongation starts immediately, indicating that phosphate is critical to the completion of postgerminative development.

Phosphate compounds which support postgerminative development. Many inorganic and organic phosphate compounds, in addition to inorganic orthophosphate meet the phosphate requirement for development subsequent to germination (table 1). The relative oxygen uptake rate is somewhat higher in the presence of Mn^{++} due to increased numbers of spores germinating and subsequently dividing. All of the compounds supported some postgerminative development as indicated, at 310 min, by the higher oxygen consumption rates accompanying cell division. However, Mn^{++} was required for typical development (table 1) of spores in the presence of glucose-1-phosphate, glucose-6-phosphate, trimetaphosphate, and tripolyphosphate. With no Mn^{++}, postgerminative development with these compounds is delayed until approximately 290 min, when there is a slight increase in the respiratory rate, and a few cells elongate and begin to divide. Other metals can substitute for Mn^{++}; Zn^{++} being effective at the same concentration, 0.4 mM; and Mg^{++} at a higher concentration, 10 mM.

The utilization of various phosphate compounds was determined by an estimation of the disappearance of these compounds from the medium during postgerminative development (table 2). Mn^{++} appears to be required for the utilization of, as well as for the development of, spores with trimetaphosphate, tripolyphosphate, and glucose-1-phosphate. Pyrophosphate can be used without Mn^{++}, but its utilization is increased when Mn^{++} is present. With Mn^{++}, all the orthophosphate, but only 30 to 58 per cent of the other compounds is consumed in 400 min.

Varying amounts of orthophosphate and of pyrophosphate are utilized in the stages of postgerminative development (figure 4). Very little phosphate uptake occurs during germination and swelling, more during elongation, and uptake increases rapidly during cell division. Mn^{++} has little effect upon the uptake of orthophosphate, but it does increase the rapidity and extent of pyrophosphate utilization.

Involvement of phosphatases in utilization of phosphates during postgerminative development. The requirement for metal activation in utilization of several of these compounds in postgerminative development suggests the possibility of the involvement of enzymatic action by phosphatases. If these compounds do serve as phosphatase substrates, orthophosphate production from them should be demonstrable. No orthophosphate can be detected in the medium when spores undergo postgerminative development with these phosphates. Possibly any orthophosphate produced is immediately taken up and utilized by the developing cell. Demonstration of the involvement of phosphatase activity in the utilization of these phosphate compounds was approached in two ways: (a) by inhibiting phosphatase activity, but not postgerminative development; (b) by inhibiting postgerminative development, in which phosphate uptake occurs, but not phosphatase activity, so that orthophosphate may accumulate and be measured.

[1] Exact time of these morphological and respiratory changes varies slightly from experiment to experiment. Occasionally, emergence occurs as late as 150 min and cell division begins as early as 210 min.

TABLE 1

*Effect of phosphate source and of Mn^{++} on postgerminative development of spores, as reflected in relative rates of oxygen consumption**

Phosphate Source	Relative O_2 Uptake Rate			
	70 min		310 min	
	K_2SO_4	$MnSO_4$	K_2SO_4	$MnSO_4$
None	1.0	1.5	1.7	2.3
Potassium orthophosphate	1.2	1.7	6.2	7.3
Sodium pyrophosphate	1.1	1.3	5.8	6.9
Fructose-1,6-diphosphate	1.0	1.5	5.1	6.5
Adenylic acid	1.4	1.9	5.1	7.2
Glycerophosphate	0.8	1.2	4.6	6.2
Creatine phosphate	1.2	—	4.9	—
Phenyl phosphate	1.1	1.7	5.2	6.3
Fructose-6-phosphate	1.1	1.4	5.5	6.8
Adenosine triphosphate	1.0	1.3	3.9	5.7
Sodium trimetaphosphate	1.0	1.4	2.6	6.3
Sodium tripolyphosphate	1.1	1.4	2.5	7.0
Glucose-1-phosphate	1.1	1.2	2.1	6.5
Glucose-6-phosphate	1.0	1.5	2.4	4.8

* Spores incubated in cacodylate buffer, pH 7.2; with glucose; and K_2SO_4, or $MnSO_4$. Phosphate sources added to give a concentration of 1.0 mM, based upon phosphorus content. Relative rate of oxygen consumption of 1.0 is equivalent to an observed rate of 0.85 μl of oxygen per min.

TABLE 2

*Utilization of phosphate compounds**

Phosphate Source	Phosphate Utilized	
	K_2SO_4	$MnSO_4$
	%	%
Potassium orthophosphate	95	100
Sodium pyrophosphate	30	47
Sodium trimetaphosphate	4	30
Sodium tripolyphosphate	7	58
Glucose-1-phosphate	0	42.5

* Spores incubated in cacodylate buffer, pH 7.2; with glucose; and K_2SO_4, or $MnSO_4$. Phosphate sources added to give a concentration of 1.0 mM, based upon phosphorus content. Reaction time, 400 min.

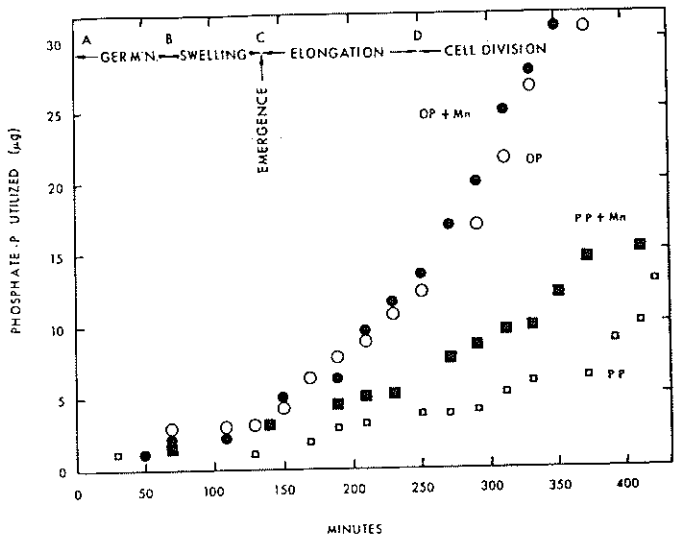

Figure 4. The utilization of orthophosphate and of pyrophosphate, with or without Mn^{++}, during germination and postgerminative development of spores of *Bacillus megaterium*. Reaction systems contained cadodylate buffer (pH 7.2); glucose; and K_2SO_4 or $MnSO_4$. Phosphates used at 1.0 mM of P.

In the first case, if phosphatase activity is necessary for the utilization of these compounds, and if this enzyme is inhibited, postgerminative development should occur only in the presence of orthophosphate. Spores were preincubated for 100 min in cacodylate buffer, glucose, and $MnSO_4$ before the addition of various chemicals described as enzyme inhibitors, and phosphate source. Pyrophosphate and glucose-1-phosphate were tested with all the inhibitors, and several of the other phosphate compounds were occasionally used. None of the chemicals (table 3, first group) inhibited postgerminative development in pyrophosphate or glucose-1-phosphate, if it occurred in the presence of orthophosphate. Attempts to inhibit phosphatases and not postgerminative development, by heat or by treatment with dilute acid, also proved unsatisfactory in that any treatment permitting postgerminative development in orthophosphate, also permitted it in the presence of the other phosphate substrates.

The second method employed to demonstrate phosphatase activity was to inhibit postgerminative development and phosphate uptake, but not phosphatase, so that orthophosphate resulting from hydrolysis of phosphate substrates could accumulate and be measured. Orthophosphate production could be demonstrated in significant quantities, but only from pyrophosphate, in the presence of the inhibitors: cysteine, sodium diethyldithiocarbamate, sodium barbital, and glycylglycine (table 3, second group). Postgerminative development and phosphate uptake are also inhibited under acid conditions, and when the pH is lower than 6.4, orthophosphate production from pyrophosphate can be shown without the addition of inhibitors. Figure 5 represents the hydrolysis of pyrophosphate by germinated spores over a 4.5-hr period. These cells appear to have several pyrophosphate hydrolyzing enzymes. Hydrolysis is activated by Co^{++} at low pH; by Mn^{++} near neutrality; and in the absence of metal ions there is little activity until a pH level greater than 8.0 is reached. Metal ions may actually be somewhat inhibitory at the higher pH levels.

Under optimal conditions, pyrophosphate can be completely hydrolyzed by germinated spores. However, we have been able to show only partial hydrolysis of phenyl phosphate, tripolyphosphate, adenylic acid, and adenosine triphosphate. Spores incubated under 99.996 per cent nitrogen produce measurable orthophosphate from pyrophosphate and from tripolyphosphate. Under these anaerobic conditions, spores germinate, but postgerminative development does not occur and orthophosphate is not taken up. We were unable, using a variety of methods, to demonstrate any production of orthophosphate by hydrolysis of glucose-1-phosphate, glucose-6-phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, or trimetaphosphate.

TABLE 3

*Effect of inhibitors on development of germinated spores, incubated with various phosphate sources; and on production of detectable orthophosphate from pyrophosphate**

Inhibitor	Concentration	Cell Division with Orthophosphate, Pyrophosphate, and Glucose-1-phosphate	Detectable Orthophosphate from Pyrophosphate
	mM		
Sodium molybdate	0.01, 0.1, 1.0 and 10.0	+	–
Sodium tungstate	0.01, 0.1, and 1.0	+	–
Potassium iodoacetate	5.0	+	–
Sodium fluoride	10.0	+	–
Thiamin	0.1 and 1.0	+	–
Urethane	1.0 and 10.0	+	–
Thiourea	1.0 and 10.0	+	–
Cysteine	1.0	+	–
Cysteine	10.0	–	+
Dinitrophenol	5.0	–	–
Sodium azide	10.0	–	–
Ethylenediaminetetraacetic acid†	0.4	–	–
Sodium diethyldithiocarbamate	1.0	–	+
Sodium barbital	50.0	–	+
Glycylglycine	50.0	–	+
Potassium cyanide	10.0	–	no test
Sodium arsenate	10.0	–	interferes with test

* Spores were germinated for 100 min, in cacodylate buffer, pH 7.0, glucose and $MnSO_4$. Inhibitor and phosphate (at a concentration of 1.0 mM of phosphorus) were added and the reaction allowed to proceed for an additional 310 min + indicates that cell division does occur, or that orthophosphate production can be measured. – indicates that cell division does not occur, or that orthophosphate production cannot be measured.

† Adenylic acid and fructose-1,6-diphosphate support postgerminative development in the presence of ethylenediaminetetraacetic acid.

DISCUSSION

There is increasing evidence that the transition of a resting bacterial spore into a rapidly metabolizing vegetative cell involves complex stepwise changes in metabolic pathways. The data reported here add further experimental support to the postulate that the nutritional requirements for germination are different from those for the subsequent metamorphosis to vegetative cell (postgerminative development).

The addition of orthophosphate in concentrations between 0.5 and 1.0 mM, is sufficient to permit further development of spores of *Bacillus megaterium*, germinated in glucose. Although these spores contain about 2.5 per cent total phosphorus, this phosphorus is either completely utilized during germination, or is in a form unavailable for the development of the germinated spore. Since vegetative cells contain twice as much phosphorus as do spores (Curran *et al.*, 1943), it is not surprising that added phosphorus is required for postgerminative development. Hachisuka *et al.* (1956), have also reported differences in the phosphate requirement for glucose oxidation in germinating spores and vegetative cells of *Bacillus subtilis*. Knaysi (1945, 1948) referring to germination in *Bacillus mycoides*, (*Bacillus cereus* var. *mycoides*) as the entire process from spore to vegetative cell, has noted the accelerating effect of the addition of phosphate on this development, and has commented on the high proportion of spores in glucose alone which become deeply stainable (our "germination"), but do not vegetate.

Very little phosphate uptake occurs during

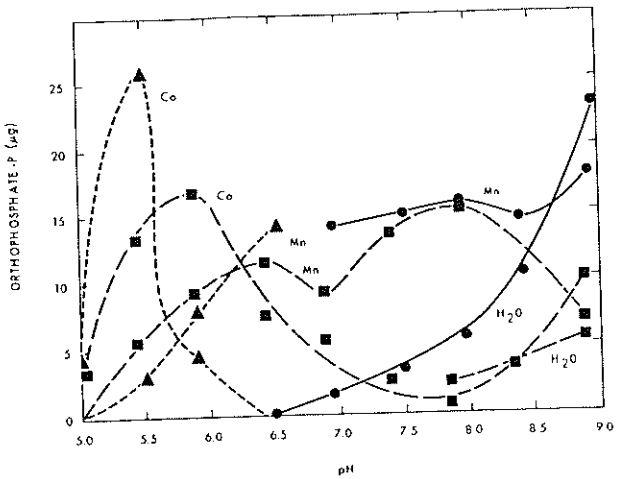

Figure 5. Hydrolysis of pyrophosphate to orthophosphate by germinated spores of *Bacillus megaterium* as a function of pH, under varying conditions. Spores (1 mg per ml) were germinated for 2 hr in barbital or cacodylate buffer at pH 6.8, with glucose and K_2SO_4. Pyrophosphate (0.5 mM), DDC (1.0 mM), and $CoSO_4$ or $MnSO_4$ (0.4 mM), when used, were added after adjustment of germinated spores to the desired pH. Data are given as μg of orthophosphate produced 4.5 hr after the addition of pyrophosphate. Legend: barbital buffer, ——; sodium diethyldithiocarbamate (DDC) in cacodylate buffer — — —; cacodylate buffer at low pH, - - - - -.

germination and swelling, more during elongation, and uptake increases rapidly during cell division. Phosphate utilization and oxygen consumption curves appear to be related in that the slopes of both curves increase after emergence, during elongation, and again during cell division. The significance of this relationship is uncertain, as the oxygen consumption curve represents rate, and the phosphate utilization curve represents total phosphate consumption. However, the data of figure 4 do suggest a marked increase in phosphate utilization rate with each change in developmental phase.

Many inorganic and organic phosphate compounds are capable of supporting postgerminative development of spores of *B. megaterium*. We have considered the possibility that these phosphate compounds are made available for postgerminative development through the mediation of phosphatases, with consequent production and utilization of orthophosphate. However, we have been unable to demonstrate the production of orthophosphate from many phosphate compounds, which do, nevertheless, support postgerminative development. It is possible that the inhibitors which we have used to prevent postgerminative development and phosphate uptake, also inhibit the particular phosphatases under investigation.

Pyrophosphate is hydrolyzed by extracts of spores, germinated spores, and vegetative cells of *B. megaterium* (Levinson *et al.*, 1958), and we have now shown that intact germinated spores produce orthophosphate from pyrophosphate, and to a lesser extent from phenyl phosphate and tripolyphosphate. However, the mere presence of pyrophosphatase is not conclusive evidence that this enzyme plays a role in either phosphate uptake or in postgerminative development. Yeast phosphatases, for example, are said to have no part in the uptake of phosphate or of sugar (Rothstein and Meier, 1949). Indeed, postgerminative development and pyrophosphatase activity of germinated spores of *B. megaterium* have different metal activation and pH requirements. At neutrality, with pyrophosphate as the phosphate source, postgerminative development proceeds in the absence of Mn^{++}. However, the pyrophosphatase of germinated spores at this pH requires metal activation. Although it may be possible to link pyrophosphatase activity and the utilization of pyrophosphate in postgerminative development, we lack evidence associating other phosphatases with this development.

More than one mechanism may be involved in the utilization of various phosphate compounds in postgerminative development of spores of *B. megaterium*. Orthophosphate and phosphate compounds hydrolyzable by intact germinated spores, may act by supplying orthophosphate, which could be incorporated into phosphorylated compounds capable of penetrating the cell membrane (Rosenberg and Wilbrandt, 1952; Rothstein, 1954). Phosphate compounds from which no orthophosphate production can be detected, but which nevertheless are utilized in postgerminative development, may act, by transfer of phosphate to glucose or unknown compounds, in such a form as to permit penetration of the cell membrane. This would make phosphate available to the cell by a mechanism not necessarily involving phosphatase.

The oxygen and pH requirements for germination differ from those for postgerminative development.

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SUMMARY

Spores of *Bacillus megaterium* germinate, but do not develop, in the absence of phosphate. The addition of orthophosphate, at a minimum concentration of 0.5 to 1.0 mM, supports postgerminative development of over 90 per cent of the germinated spores in a suspension containing approximately 4.2×10^8 spores per ml. Many other inorganic and organic phosphate compounds also permit this development, and some of these compounds are more readily utilized in the presence of metal activators, such as Mn^{++}.

Germinated spores hydrolyze pyrophosphate to orthophosphate, but this can be demonstrated only under conditions inhibiting postgerminative development and phosphate uptake. Metal activation and pH data suggest the presence of more than a single pyrophosphatase. We have been unable to demonstrate hydrolysis of many other phosphorylated compounds, which do, however, support postgerminative development.

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